Biochemical Pharmacology, Vol. 19, pp. 457–472. Pergamon Press. 1970. Printed in Great Britain

EFFECTS OF STORAGE ON HEPATIC MICROSOMAL CYTOCHROMES AND SUBSTRATE-INDUCED DIFFERENCE SPECTRA*

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(*Received* 24 *March* 1969; *accepted* 6 *June* 1969)

Abstract—Microsomal cytochrome P450, the ethyl isocyanide difference spectrum of dithionite-reduced microsomes (measured at the pH giving equal absorbance of 430 and 455 mμ peaks), and the aniline type II difference spectrum were affected similarly after phenobarbital induction and during the subsequent aging *in vitro* (about 90 hr at 1° under nitrogen) of hepatic microsomes from rats pretreated with phenobarbital. In these same microsomal preparations from phenobarbital-pretreated rats, type I spectra (caused by adding hexobarbital or (+) benzphetamine to microsomes) were increased to a greater extent and were less stable to aging *in vitro* than the previously mentioned components.

During aging, decreases in the type II spectra paralleled the loss of total heme, but large decreases in the type I spectra were observed with little or no loss of heme. Also, aging did not change the pH giving equal absorbance of the 430 and 455 mμ peaks in the ethyl isocyanide difference spectrum, nor was there any shift in the wavelength of the maximum (450 mμ) in the carbon monoxide difference spectrum of dithionite-reduced microsomes.

The binding associated with type I or II spectral changes did not appear to conform to simple Michaelis–Menten kinetics. Plots of ΔA vs. ΔA/S (where ΔA is the difference in absorbance between the trough and the peak in the 350 to 450 mμ region of the type I or II substrate-induced microsomal difference spectrum, and S is the corresponding substrate concentration) exhibited curvature (concave upwards). During aging, the curvature associated with plots of ΔA vs. ΔA/S using (+) benzphetamine increased, while the curvature associated with plots of ΔA vs. ΔA/S using aniline decreased.

Some correlation was observed between increases in the rate of *N*-demethylation of (+) benzphetamine or *p*-hydroxylation of aniline and the increases in the respective type I or II difference spectra, after phenobarbital treatment of rats. Also it appeared that the rate of *p*-hydroxylation of aniline decreased to a lesser extent on aging than the rate of *N*-demethylation of (+) benzphetamine.

CARBON monoxide[1–3] and ethyl isocyanide[4] give characteristic difference spectra with dithionite-reduced hepatic microsomes. These difference spectra have been associated with reagent binding to cytochrome P450.[3,4] This pigment, in contrast to cytochrome b_5

* This research was supported by a grant (# GM12675) from the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland.

† Recipient of a Wellcome Travelling Fellowship from the Wellcome Trust.

(the other major cytochrome present in hepatic microsomes), apparently mediates many oxidative and reductive drug biotransformations.[5-7]

Recently, it has been shown that a wide variety of substrates and inhibitors of cytochrome P450-mediated hepatic drug metabolism cause essentially two types of difference spectra when added to nonreduced hepatic microsomes.[8, 9] The type I difference spectrum is characterized by a trough at about 420 mμ and a peak at about 385 mμ, whereas the type II difference spectrum is characterized by a peak at about 430 mμ and a trough at about 395 mμ. Both these spectral changes, it is suggested,[8, 9] involve interaction with cytochrome P450.

The proposed binding associated with type I and II difference spectra appeared to obey Michaelis–Menten kinetics, and the substrate concentration required for half-maximal spectral change was defined as the "spectral dissociation constant" or "K_s".[8]

In the present study, we have compared the stabilities, during storage of microsomes, of several drug-metabolizing enzyme activities and microsomal spectral components because this knowledge is desirable for practical purposes, and it may enable more of the origin of these spectra and the binding sites involved to be ascertained.

MATERIALS AND METHODS

Animals. Male, Long–Evans rats (150–250 g) were used in all experiments and were allowed free access to laboratory chow and water up to the time of sacrifice. Phenobarbital pretreatment comprised three consecutive daily i.p. injections of phenobarbital sodium (75 mg/kg body weight); the last injection was made approximately 24 hr prior to sacrifice.

Microsomal preparation. The rats were killed by cervical dislocation and the livers immediately perfused with 0·9% (w/v) NaCl. All subsequent steps were performed at 0 to 4°. The livers were homogenized (Potter homogenizer with plastic pestle) with 2 vols. of 1·15% (w/v) KCl, and cell debris, nuclei and mitochondria removed by 20-min centrifugation at 9000 *g* in a B20 International ultracentrifuge. The supernatant was centrifuged for 60 min at 105,000 *g* in an L2 65B Beckman ultracentrifuge, and the microsomal pellet was suspended in 0·1 M phosphate buffer (pH 7·4) and recentrifuged at 105,000 *g* for 45 min. The washed microsomal pellet was suspended in 0·1 M phosphate buffer (pH 7·4) to give a protein concentration of 10–15 mg/ml. Protein was assayed by a micro-Kjeldahl technique.[10]

Storage of microsomes. The microsomal suspension, as prepared above, was bubbled for 20 sec with nitrogen and stored in a stoppered flask at 1° in the absence of light. At time intervals indicated in the text, samples were removed and diluted for spectral measurements and determination of enzymic activity. Before sampling, the entire flask contents were thoroughly mixed, and after sampling the vessel was reflushed with nitrogen, resealed and returned to the refrigerator.

Assays for microsomal drug-metabolizing enzyme activity. Incubations were carried out in a Dubnoff shaking apparatus (100 oscillations/min) at 37° under an atmosphere of oxygen (flow rate 1000 ml/min). A glass marble (diameter 15 mm) was added to each incubation beaker (capacity 15 ml) to facilitate mixing of the contents during incubation.

Incubation media contained glucose 6-phosphate, 7 mM; $MgSO_4$, 5 mM; semicarbazide, 10 mM (for formaldehyde assays only); glucose 6-phosphate dehydrogen-

ase, 3 units;* drug substrate [(+) benzphetamine,† 1·4 mM; or aniline, 2·5 mM]; microsomes equivalent to 6 to 8 mg protein; phosphate buffer (pH 7·4), 80 to 100 mM; NADP, 1·4 or 4·2 mM (for (+) benzphetamine or aniline metabolism respectively); in a volume of 5 ml. The incubation media minus NADP were prepared at 0 to 4°. The NADP was added to the incomplete media after they were warmed to 37° by 5-min preincubation. Subsequent incubation was for 15 min [(+) benzphetamine] or 20 min (aniline).

The *N*-demethylation of (+) benzphetamine was measured by the formaldehyde produced in the reaction. The demethylation reaction was terminated by the addition of 10 ml 0·6 M perchloric acid to 5 ml of reaction medium. After removal of the protein by centrifugation, 3 ml of the supernatant was mixed with 2 ml of double-strength Nash reagent B[11] and incubated at 60° for 30 min in a water bath. After cooling to room temperature, the intensity of the yellow color was read at 415 mμ. Formaldehyde produced by reactions not involving drug substrate was corrected for by performing incubations from which only (+) benzphetamine was omitted.

Aniline hydroxylase activity was determined by measuring *p*-aminophenol formation.[12]

Measurement of microsomal spectra. All spectra were recorded using a Shimadzu model MPS 50L recording spectrophotometer with the samples kept at room temperature. The carbon monoxide-difference spectra of dithionite-reduced microsomes were obtained essentially as described by Omura and Sato;[3] cytochrome P450 was determined from the difference in absorbance between 450 and 490 mμ. The ethyl isocyanide difference spectra of reduced microsomes were obtained as described by Sladek and Mannering;[13] the differences in absorbance between 430 and 490 mμ and between 455 and 490 mμ were measured. Except where otherwise indicated in the text, final microsomal protein concentrations of 1·5 to 3·0 mg per ml were used for spectral studies. The spectral measurements were linear with protein concentration up to at least 3 mg protein/ml. For the determination of ethyl isocyanide spectra, the microsomal suspensions were diluted with 1 M potassium phosphate buffer (pH 7·4). For all other difference spectra, 0·1 M potassium–sodium-phosphate buffer (pH 7·4) was used.

Type I and II difference spectra (in the presence of substrates) were obtained using nonreduced microsomes.[8] Solutions of (+) benzphetamine, hexobarbital or aniline were added to 3 ml of microsomal suspension by micropipette. With each addition to the sample cuvette a corresponding volume of water was added to the reference cuvette. The maximum volume added per cuvette was 0·06 ml. The difference in absorbance between the trough and peak in the 350 to 450 mμ region was measured.

The cytochrome b_5 and total heme contents of microsomes were determined as described by Omura and Sato.[3] For cytochrome b_5 the difference in absorbance between 424 and 409 mμ was measured and an extinction coefficient of 185 $cm^{-1}mM^{-1}$ was used, while for heme the difference in absorbance between 557 and 575 mμ was measured and the extinction coefficient was 32·4 $cm^{-1}mM^{-1}$.

Preparation of hemoglobin and methemoglobin. Hemoglobin was prepared from rat erythrocytes essentially as described by St. George and Pauling.[14] The concentration

* One unit of glucose 6-phosphate dehydrogenase is the amount of enzyme that reduces 1 μmole of NADP per min at pH 7·4 and 25°.

† (+) *N*-Benzyl-*N*, α-dimethylphenethylamine.

of hemoglobin was determined from the carbon monoxide difference spectrum, measuring the difference in absorbance between 420 and 490 mμ and using an extinction coefficient of 93 $cm^{-1}mM^{-1}$.[15] Methemoglobin was prepared by the ferricyanide oxidation of hemoglobin. For quantitation, the methemoglobin was reduced by dithionite to hemoglobin.

Microsomal contamination with hemoglobin and/or methemoglobin. The carbon monoxide difference spectrum of dithionite-reduced microsomes exhibited a slight shoulder in the 420 mμ region, which was not removed by washing the microsomes. This shoulder was still observed even though the carbon monoxide difference spectrum of nonreduced microsomes showed no carboxyhemoglobin peak at 420 mμ. We concluded that our nonreduced microsomal preparations might contain very small amounts of hemoglobin. The shoulder in the 420 mμ region in the carbon monoxide difference spectrum of reduced microsomes may also have been caused by cytochrome P420[3] and/or methemoglobin originally present in the nonreduced preparations. Although the spectrum of nonreduced microsomes showed no decrease in absorbance at 634 mμ on the addition of solid sodium cyanide (indicating the absence of methemoglobin) it is possible that this method (after Michel and Harris[16]) would not detect the very small amounts of methemoglobin which would cause the shoulder described above.

Kinetic studies of binding associated with substrate-induced difference spectra. The data was plotted analogously to the method of Hofstee.[17] That is, the magnitude of the type I or II difference spectrum (ΔA) was plotted versus ΔA/S, where S was the corresponding drug substrate concentration. This method of plotting was chosen for the following reasons:

(1) It appears superior to the double reciprocal plot (Lineweaver–Burk) for the determination of kinetic constants if simple Michaelis–Menten kinetics were followed and slopes of regression lines were calculated using the method of least squares.[18]

(2) The method appears sensitive for detecting deviations from simple Michaelis–Menten kinetics.[19]

Regression lines were fitted to the ΔA vs. ΔA/S plots by the method of least squares using a computer program.[20]

RESULTS

The microsomal difference spectrum caused by adding (+) benzphetamine. The addition of (+) benzphetamine to male rat hepatic microsomes (aerobic) gave a type I difference spectrum qualitatively identical with that produced using hexobarbital* (peak and trough at 385 and 420 mμ respectively). The type I difference spectrum due to (+) benzphetamine or hexobarbital was displaced on the addition of "type II" substrates such as aniline or nicotinamide to give a resultant type II spectrum. This is in general agreement with the findings of McLean and Schenkman *et al.*[21, 22]

Hexobarbital and (+) benzphetamine gave near-maximal spectral changes at concentrations of 1·0 and 0·33 mM, respectively, the spectral change due to (+) benzphetamine being 20–30 per cent greater than that due to hexobarbital. This was true using hepatic microsomes from control or phenobarbital-pretreated rats (see Table 1).

* The racemic mixture of (+) and (−) forms was used throughout this study.

TABLE 1. EFFECT OF PHENOBARBITAL PRETREATMENT ON HEPATIC MICROSOMAL SPECTRAL COMPONENTS*

Spectral component	Units	Pre-treatment	Experiment no. 1 Magnitude of component	1 Ratio of: phenobarb./control	2 Magnitude of component	2 Ratio of: phenobarb./control	3 Magnitude of component	3 Ratio of: phenobarb./control
Cytochrome P450†	mμmole per mg protein	Control	1·00		1·16		0·76	
		Phenobarb.	2·33	2·33	2·60	2·24	1·85	2·44
Type II difference spectrum (aniline 20 mM)	ΔA(430–397 mμ) per mg protein per ml	Control	0·041		0·051		0·029	
		Phenobarb.	0·087	2·12	0·094	1·84	0·069	2·38
Type I difference spectrum [(+) benzphetamine, 0·33 mM]	ΔA(385–420 mμ) per mg protein per ml	Control	0·035		0·044		0·029	
		Phenobarb.	0·123	3·54	0·143	3·25	0·098	3·38
Type I difference spectrum (hexobarbital, (1 mM)	ΔA(385–420 mμ) per mg protein per ml	Control	0·029		0·034		0·025	
		Phenobarb.	0·092	3·17	0·114	3·35	0·074	2·96
Ethyl isocyanide (3·45 mM) spectrum‡	ΔA(430 or 455–490 mμ) per mg protein per ml	Control	0·056		0·072		0·044	
		Phenobarb.	0·126	2·25	0·153	2·12	0·107	2·43
Cytochrome b_5	mμmole per mg protein	Control	0·668		0·637		0·509	
		Phenobarb.	0·750	1·12	0·800	1·26	0·580	1·14
Total heme	mμmole per mg protein	Control	1·82		2·06		1·51	
		Phenobarb.	3·12	1·71	3·46	1·67	2·65	1·71
Total heme minus cytochrome b_5 heme	mμmole per mg protein	Control	1·15		1·42		1·00	
		Phenobarb.	2·37	2·06	2·66	1·87	2·07	2·07

* For each experiment the pools of microsomes from control and phenobarbital-pretreated male rats were obtained from not less than three livers.
† The extinction coefficient of 91 cm^{-1} mM^{-1} (Omura and Sato[3]) was used to convert ΔA (450–490 mμ) into mμmoles.
‡ Difference spectrum using dithionite-reduced microsomes at pH giving equal absorbance of 430 and 455 mμ peaks.

TABLE 2. EFFECT OF PHENOBARBITAL PRETREATMENT ON THE *N*-DEMETHYLATION OF (+) BENZPHETAMINE, *p*-HYDROXYLATION OF ANILINE, CYTOCHROME P450 AND TYPE I AND II DIFFERENCE SPECTRA*

			Experiment no. 1		Experiment no. 2	
Spectral component or metabolic route	Units	Pretreatment	Magnitude of spectral component or rate of metabolism	Ratio of: phenobarb. / control	Magnitude of spectral component or rate of metabolism	Ratio of: phenobarb. / control
N-Demethylation of	mμmole HCHO per	Control	5·7		5·3	
(+) benzphetamine	mg protein per min	Phenobarbital	16·9	2·97	15·9	3·00
p-Hydroxylation	mμmole *p*-aminophenol	Control	1·0		1·2	
of aniline	per mg protein per min	Phenobarbital	1·4	1·40	2·0	1·66
Cytochrome P450†	mμmole	Control	1·18		1·27	
	per mg protein	Phenobarbital	2·17	1·84	2·62	2·07
Type II difference	ΔA(430–397 mμ)	Control	0·050		0·053	
spectrum (aniline, 20 mM)	per mg protein per ml	Phenobarbital	0·072	1·44	0·086	1·63
Type I difference spectrum	ΔA(385–420 mμ)	Control	0·041		0·047	
[(+) benzphetamine 0·33 mM]	per mg protein per ml	Phenobarbital	0·106	2·59	0·139	2·69

* For each experiment the pools of microsomes from control and phenobarbital-pretreated male rats were obtained from not less than three livers.
† The extinction coefficient of 91 cm^{-1} mM^{-1} (Omura and Sato[3]) was used to convert ΔA (450–490 mμ) into mμmoles.

The effect of phenobarbital pretreatment on microsomal spectral components and drug metabolism. To facilitate enzymic and spectral measurements, hepatic microsomes from phenobarbital-pretreated rats were used for subsequent stability studies.

Tables 1 and 2 show the effect of phenobarbital pretreatment on various microsomal spectral components and the microsomal *N*-demethylation of (+) benzphetamine and *p*-hydroxylation of aniline. Cytochrome P450 and the ethyl isocyanide difference spectrum of reduced microsomes (measured at the pH where the 430 and 455 mμ peaks were of equal absorbance) appeared to be induced to similar extents (90–150 per cent increase). The pH giving equal absorbance of the 430 and 455 mμ peaks (about pH

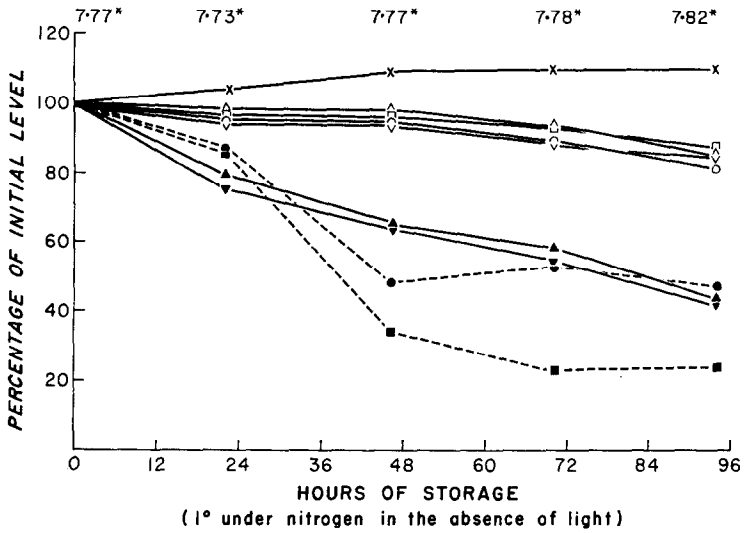

FIG. 1. The effect of storage of hepatic microsomes from phenobarbital-pretreated male rats on various spectral components and the *N*-demethylation of (+) benzphetamine and *p*-hydroxylation of aniline. The spectral components measured were cytochrome P450, □—□; ethyl isocyanide (3·45 mM) difference spectrum of dithionite-reduced microsomes (at the pH giving equal absorbance of the 430 and 455 mμ peaks), Δ—Δ; aniline (20 mM) type II difference spectrum, ○—○; (+) benzphetamine (0·33 mM) type I difference spectrum, ▲—▲; hexobarbital (1·0 mM) type I difference spectrum, ▼—▼; heme, ▽—▽; cytochrome b_5, ×—×. The drug metabolisms studied were the *N*-demethylation of (+) benzphetamine, ■—■; and the *p*-hydroxylation of aniline, ●—●; the initial levels of the spectral components are listed in Table 1 under Experiment 3 in the "Phenobarbital pretreatment" category. The initial rates of *N*-demethylation of (+) benzphetamine and *p*-hydroxylation of aniline were 12·9 mμmoles HCHO per mg microsomal protein per min and 1·25 mμmoles *p*-amino phenol per mg microsomal protein per min respectively.

* This is the pH after 0, 22, 45, 70 and 94 hr of storage, which gave equal absorbance for the 430 and 455 mμ peaks in the ethyl isocyanide difference spectrum obtained using dithionite-reduced microsomes.

7·8) did not appreciably change on phenobarbital treatment. Heme not due to cytochrome b_5, and the aniline (20 mM) type II difference spectrum, increased to a slightly lesser extent than cytochrome P450. The most marked induction was observed with the type I difference spectra caused by hexobarbital (1 mM) or (+) benzphetamine (0·33 mM), where increases of over 200 per cent were noted. The increases were similar whether the type I difference spectrum was produced by hexobarbital or (+) benzphetamine. In agreement with previous reports,[23–25] and compared with cytochrome

P450, the cytochrome b_5 content was increased to a relatively small extent (10 to 25 per cent in our studies) by phenobarbital treatment.

It is interesting to note that there appeared to be some correlation between the increases in magnitudes of type I and II difference spectra and the rates of metabolism of the corresponding substrates; the *N*-demethylation of (+) benzphetamine was induced to a significantly greater extent than the *p*-hydroxylation of aniline (Table 2).

The effect of storage on spectral components and drug metabolism using microsomes from phenobarbital-pretreated male rats. The results from a typical experiment (Fig. 1) show the effects of storage (at 1° under nitrogen in the absence of light) on various spectral components and the *N*-demethylation of (+) benzphetamine and *p*-hydroxylation of aniline, using microsomes from phenobarbital-pretreated rats. Cytochrome P450, the ethyl isocyanide difference spectrum of reduced microsomes (at the pH where 430 and 455 mμ peaks were of equal absorbance), the aniline (20 mM) type II difference spectrum, and total heme appeared on storage to decrease relatively slowly and at similar rates. With four similar experiments these components did not decrease by more than 8 per cent in 24 hr or more than 30 per cent in 90 hr.

Type I difference spectra caused by hexobarbital (1 mM) or (+) benzphetamine (0·33 mM) "decayed" at similar rates, and much more rapidly than other spectral components. The type I difference spectrum had usually decreased by 25–40 per cent after 24 hr and more than 50 per cent after 90 hr.

In no experiment did the apparent cytochrome b_5 content decrease during 90 hr of microsomal storage. In fact, rather surprisingly, in most cases 10–15 per cent increases in the absorbance associated with this component were recorded.

The metabolic measurements showed greater variability, but in nearly all cases the ability of the microsomes to demethylate (+) benzphetamine decreased more rapidly than the ability to *p*-hydroxylate aniline.

The pH value (approx. 7·8) giving equal absorbances for the 430 and 455 mμ ethyl isocyanide peaks did not appreciably change during microsomal storage, although the absolute absorbance of each peak did decrease (see Figs. 1 and 2). In addition, no change in the wavelength of the (450 mμ) maximum of the carbon monoxide difference spectrum of dithionite-reduced microsomes was detected during storage.

In no aging experiment was an increase (with time) at 420 mμ noted in the carbon monoxide difference spectrum of dithionite-reduced microsomes. This indicates that during storage either no cytochrome P420 was formed or that if it were formed it was rapidly degraded.

It has been shown,[24] using microsomes from phenobarbital-treated rabbits, that the type I difference spectrum seen after adding *N*-ethylaniline is less stable to pH change than the corresponding aniline type II difference spectrum. In the present studies, no pH change greater than 0·05 of a unit was noted in the pools of microsomes stored over a period of 90 hr, indicating that a pH change could not account for the comparatively rapid decrease of the type I difference spectrum.

This relative instability on storage of type I compared with type II difference spectra, which has also recently been noted by Shoeman *et al.*,[26] was investigated further.

Kinetic studies of binding associated with type I and II difference spectra before and after microsomal storage. Figure 3 shows ΔA plotted against ΔA/S for the aniline-

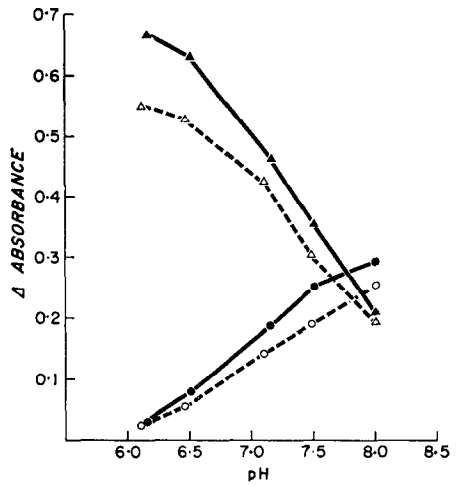

FIG. 2. The pH dependence of the 430 and 455 mμ peaks of the ethyl isocyanide difference spectrum of dithionite-reduced microsomes from phenobarbital-pretreated male rats; before and after storage The values on the ordinate represent ΔA (455 or 430 minus 490 mμ) at the microsomal protein concentration of 2·60 mg per ml. Triangles correspond to the 430 mμ peak, and circles correspond to the 455 mμ peak. Closed symbols with solid lines, and open symbols with dashed lines correspond to 0 and 94 hr of storage (at 1° under nitrogen in the dark) respectively.

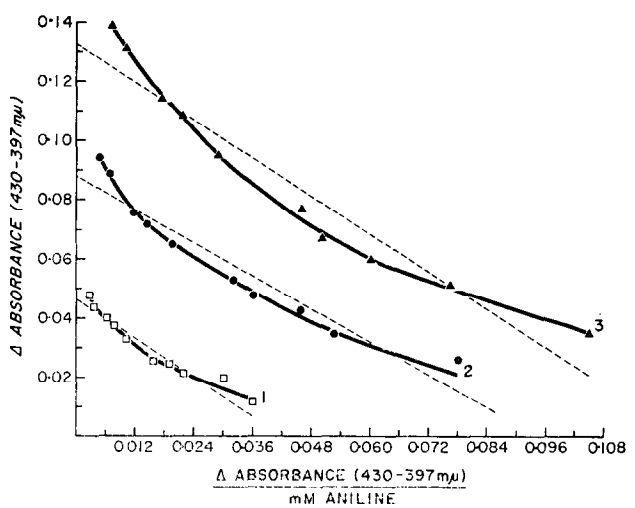

FIG. 3. Aniline (type II) spectral data plotted after the method of Hofstee.[17] Hepatic microsomes were obtained from phenobarbital-pretreated rats. The arabic numerals 3, 2 and 1 refer to different microsomal protein concentrations; concentration No. 3 was 2·00 mg per ml, concentration No. 2 was 1·33 mg per ml, concentration No. 1 was 0·67 mg per ml. The dashed regression lines were fitted to the experimental points by the method of least squares.

microsome difference spectra at three different microsomal protein concentrations. Although the lines appear almost parallel, they are nonlinear,* indicating that simple Michaelis–Menten kinetics were not followed. Corresponding plots of the Hofstee type for (+) benzphetamine-microsome difference spectra also exhibited curvature (Fig. 4). Similar results were obtained using hexobarbital. The curvature associated with the type I substrates appeared to be less marked than that seen with aniline (compare Figs. 3 and 4).

To test whether these curvilinear relationships were caused by traces of methemoglobin and/or hemoglobin bound to microsomes, aniline and (+) benzphetamine were added to model test systems of solutions of methemoglobin or hemoglobin. When

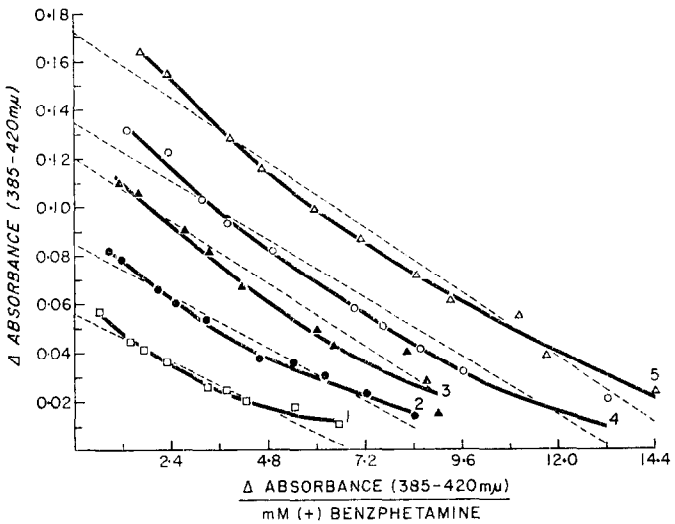

FIG. 4. (+) Benzphetamine (type I) spectral data plotted after the method of Hofstee.[17] Hepatic microsomes were obtained from phenobarbital-pretreated rats. The arabic numerals 5, 4, 3, 2, and 1 refer to different microsomal protein concentrations; concentration No. 5 was 2·00 mg per ml, concentration No. 4 was 1·50 mg per ml, concentration No. 3 was 1·33 mg per ml, concentration No. 2 was 1·00 mg per ml, and concentration No. 1 was 0·67 mg per ml. The dashed regression lines were fitted to the experimental points by the method of least squares.

aniline (20 mM) was added to a 0·006 mM hemoglobin solution (with this concentration of hemoglobin the difference between the 420 mμ peak and 490 mμ in the carbon monoxide difference spectrum was about 0·55 of an absorbance unit) a small difference spectrum was obtained with a trough at 420 mμ and a peak at 405 mμ. The difference between the peak and trough of the aniline difference spectrum was 0·02 of an absorbance unit. The results using methemoglobin were quantitatively similar, only the aniline difference spectrum showed a trough at 430 mμ and a peak at 415 mμ. However, amounts of methemoglobin and/or hemoglobin necessary to cause the slight

* The nonlinearity was not caused by a change in pH on substrate addition (addition of 20 mM aniline hydrochloride to microsomes in 0·1 M phosphate buffer lowered the pH from 7·4 to 7·0), as increasing the buffering capacity of the microsomal suspension and/or adding equimolar hydrochloric acid to the reference cuvette did not alter the curvature of the ΔA vs. ΔA/S plots.

shoulder in the 420 mμ region, which was obtained in the carbon monoxide difference spectrum of dithionite-reduced freshly prepared microsomes, gave no measurable difference spectrum with 20 mM aniline (difference in absorbance between trough and peak less than 0·002 of an absorbance unit). It was not possible to obtain a difference spectrum by adding (+) benzphetamine to hemoglobin or methemoglobin solutions. Therefore, it seems unlikely that the non-Michaelis–Menten kinetics associated with the substrate-induced difference spectra, caused by adding either (+) benzphetamine or aniline to microsomes, can be attributed to the presence of traces of methemoglobin or hemoglobin in the microsomes.

As shown in Fig. 5, in the case of aniline, storage of microsomes at 1° in the dark under nitrogen appeared to decrease the curvature of the ΔA vs. ΔA/S plots. This is further illustrated in Table 3 by the increase (with storage) of the correlation coeffi-

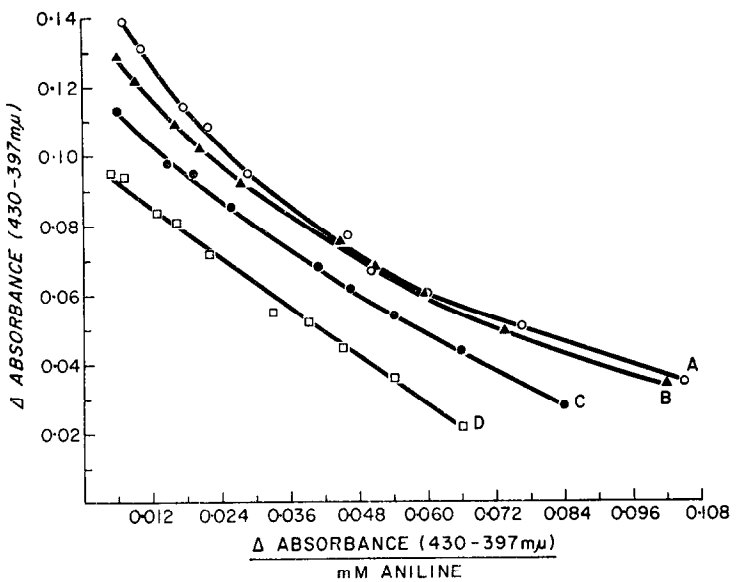

FIG. 5. The effect of microsomal storage on aniline (type II) spectral data plotted after the method of Hofstee.[17] Hepatic microsomes were obtained from phenobarbital-pretreated rats. Spectra were measured at a microsomal protein concentration of 2·00 mg per ml. A, B, C, and D, respectively, correspond to 0, 21, 45, and 69 hr of storage at 1° under nitrogen in the dark.

cients for the "best" straight lines fitted to the experimental points. The slopes of the regression lines of ΔA vs. ΔA/S with aniline did not markedly change during 69 hr of storage (Table 3).

In contrast, for (+) benzphetamine the curvature of the ΔA vs. ΔA/S plots appeared to increase with microsomal storage (Fig. 6). These experiments were repeated using several concentrations of microsomes and the results were similar to those seen in Fig. 6. Microsomal protein concentrations varied from 0·67 to 2·00 mg per ml. This same kind of storage-induced increase in curvature of ΔA vs. ΔA/S plots occurred using hexobarbital as the type I substrate.

TABLE 3. REGRESSION ANALYSIS OF ANILINE SPECTRAL DATA (ΔA VS. ΔA/S PLOT) OBTAINED BEFORE AND DURING MICROSOMAL STORAGE*

Hours of storage (at 1° under N_2 in the dark)	Microsomal protein (mg per ml)	Regression coefficient ("slope") for ΔA vs. ΔA/S (mM)†	"ΔA_{max}" (intersection of regression line with vertical axis)†	Correlation‡ coefficient for ΔA vs. ΔA/S
0	2·00	−1·07	0·133	0·9627
	1·33	−0·93	0·088	0·9545
	0·67	−0·98	0·046	0·9670
21	2·00	−1·00	0·125	0·9781
	1·33	−1·03	0·087	0·9801
	0·67	−1·00	0·042	0·9713
45	2·00	−1·09	0·116	0·9957
	1·33	−1·07	0·077	0·9957
	0·67	−1·11	0·035	0·9932
69	2·00	−1·21	0·100	0·9963
	1·33	−1·12	0·060	0·9975

* ΔA is the difference in absorbance between the 430 mμ peak and 397 mμ trough of the aniline (type II) hepatic microsomal difference spectrum. S is the corresponding concentration (mM) of aniline. Microsomes from phenobarbital pretreated male rats were used.

† The slopes and intercepts of the regression lines were determined by the method of least squares.

‡ The correlation coefficient is a measure of the extent to which the plotted data fit a straight line. The nearer the correlation coefficient approaches 1 the more closely ΔA vs. ΔA/S conforms to a linear relationship.

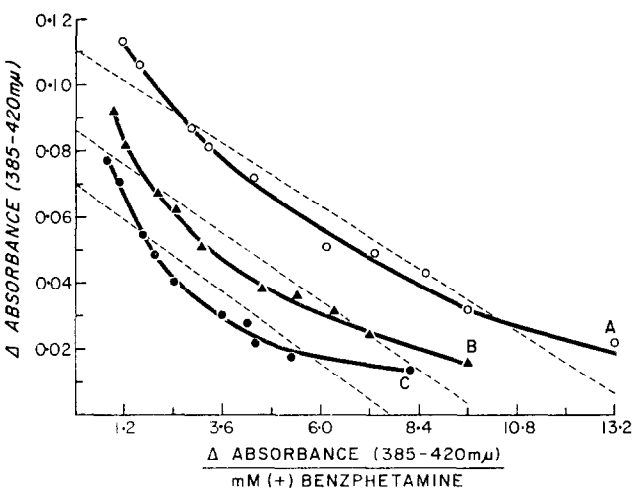

FIG. 6. The effect of microsomal storage on (+) benzphetamine (type I) spectral data plotted after the method of Hofstee.[17] Hepatic microsomes were obtained from phenobarbital-pretreated rats. Spectra were measured at a microsomal protein concentration of 2·00 mg per ml. A, B, and C, respectively, correspond to 0, 21, and 45 hr of storage at 1° under nitrogen in the dark. The dashed regression lines were fitted to the experimental data by the method of least squares.

DISCUSSION

It appears that cytochrome P450, the ethyl isocyanide difference spectrum of dithionite-reduced microsomes (measured at the pH where the 430 and 455 mμ peaks are of equal absorbance), and the aniline type II difference spectrum are affected similarly after phenobarbital treatment of animals and after the subsequent aging *in vitro* of hepatic microsomes from animals thus induced.

Nishibayashi *et al.*[4] correlated cytochrome P450 content of a variety of tissues from control and phenobarbital-pretreated animals with the magnitudes of the two ethyl isocyanide peaks obtained with dithionite-reduced preparations, and with the magnitude of the single 434 mμ peak with ethyl isocyanide-treated nonreduced microsomes. However, the degree of correlation was noted to be less with the reduced than with the nonreduced preparations. Lange[24] found that the magnitudes of the 434 and 430 mμ ethyl isocyanide peaks paralleled the cytochrome P450 increase which occurred after phenobarbital treatment of rabbits, but also found that the 455 mμ ethyl isocyanide peak was increased to a greater extent than the other peaks after phenobarbital treatment.

Correlations between cytochrome P450 content and the maximal type II difference spectrum caused by aniline have been demonstrated using different batches of control rabbit liver microsomes,[9] rabbit liver microsomes before and after phenobarbital treatment,[24] and liver microsomes from male and female rats.[27] Also, it has been shown in this laboratory* that benzpyrene pretreatment in rats caused a shift (about 2 to 4 mμ) of the 430 mμ peak, in the aniline type II spectrum, to a shorter wavelength. This seems analogous to the shift of the 450 mμ peak to 446 mμ as seen in the carbon monoxide difference spectrum of dithionite-reduced microsomes after pretreatment of rats with 3-methylcholanthrene.[28]

Further evidence which suggests that aniline, ethyl isocyanide and carbon monoxide all react with the same microsomal component is the spectral competition between: (a) carbon monoxide and ethyl isocyanide with dithionite-reduced microsomes,[3] or (b) aniline and carbon monoxide with NADPH-reduced microsomes,[8] or (c) ethyl isocyanide and aniline with nonreduced microsomes.[29] Also relevant is the fact that ethyl isocyanide and aniline appear to give similar difference spectra with dithionite-reduced[30] and nonreduced[8] hepatic microsomes. However, the aniline-induced spectral changes are markedly smaller in both cases than those caused by ethyl isocyanide. From kinetic evidence Imai and Sato[29] deduced that, with nonreduced microsomes, ethyl isocyanide and aniline bind to different sites on cytochrome P450, but that these sites are sufficiently close to enable each of these substrates to interfere with the binding of the other.

Our studies demonstrated that not only was the type I difference spectrum (seen after adding hexobarbital or (+) benzphetamine) more susceptible to phenobarbital induction, it was also less stable to aging *in vitro* than the type II difference spectrum and other spectral components. Figure 1 indicates that the decay of aniline, ethyl isocyanide or carbon monoxide-difference spectra was proportional to the loss of heme. In contrast, marked decreases in the magnitude of the type I difference spectrum were observed with little or no concomitant loss of heme. This finding, together with the reported inability of type I compounds to displace carbon monoxide from cytochrome P450,[8] although basic amines can do so,[3, 8] indicates that type I compounds

* D. R. Rickert, unpublished observation.

do not bind to the heme portion of the cytochrome P450 molecule. However, the fact that type II substrates can displace a type I difference spectrum, possibly by an allosteric mechanism, suggests that type I and II substrate binding sites exist on the same cytochrome P450 molecule.

Current ideas concerning the origin of type I and II difference spectra implicate cytochrome P450 as the microsomal component involved. Schenkman *et al.*[8] favored the idea that type I and II substrates react with cytochrome P450 to modify the heme protein–ligand interaction in different ways. Imai and Sato[9] agreed with this general hypothesis and suggested that the modification of the ligand interaction may be a result of substrate binding to the nonheme (particularly the protein) moiety of cytochrome P450. Imai and Sato[9] proposed this idea since many substrates (notably type I) are unsuitable as ligands for the heme iron. Recently Schenkman and Sato,[31] correlating from a model system using ferriheme solutions, suggested that the interaction of type I substrates with microsomes causes alterations in the electronegativity of the sixth ligand of the heme of cytochrome P450, which result in the production of the characteristic spectral change.

Assuming that substrate binding is associated with type I and II spectral changes, it is apparent that in contrast to reports of several other workers,[8, 9, 27] our data are not consistent with the binding conforming to simple Michaelis–Menten kinetics. This discrepancy may be due to the facts that: (1) we used wider ranges of substrate concentrations than in some of the previously published reports, and (2) the method of double reciprocal plotting (used by previous workers) may be less sensitive for detecting deviations from simple kinetics than the method we used. However, Imai and Sato,[9] with regard to aniline-induced spectral changes and the subsequent reciprocal plotting of data, do mention that, "The experimental points were not strictly on a straight line . . .". It is also interesting that Wada *et al.*[32] obtained an analogous curvilinear relationship by plotting reciprocals of the rate of *p*-hydroxylation of aniline vs. aniline concentration using hepatic microsomes from control rats, control mice and phenobarbital-pretreated mice. However, a linear relationship appeared to apply with phenobarbital-pretreated rats when 1/V vs. 1/S was plotted with aniline as substrate of the microsomal system.[32]

The nonlinearity of the ΔA vs. $\Delta A/S$ plots as described in this study is suggestive of more than one binding site for both type I and II substrates. In the case of aniline the data would be compatible with the idea of a di-heme cytochrome P450 molecule as postulated by other workers.[31, 33, 34]

It has already been indicated that small amounts of methemoglobin, and/or hemoglobin which might be present in the microsomes, did not give any significant spectral changes with either aniline or benzphetamine. Therefore we believe the curvature in ΔA vs. $\Delta A/S$ plots cannot be attributed to methemoglobin and/or hemoglobin. It is unlikely that cytochrome P420, if present only in trace amounts, would cause the marked curvature associated with the aniline spectral data (Fig. 3). This is concluded since Imai and Sato[9] have reported that with cytochrome P420, 20 mM aniline (the maximum concentration used in our study) gives a difference spectrum of only 25 per cent of the magnitude of that produced with an equal amount of cytochrome P450. These workers reported the dissociation constant of the aniline-cytochrome P420 complex was 100 mM.

Even when only one type of binding site is involved, two alternative substrates

present in the system can result in a ΔA vs. ΔA/S plot which is concave upwards or alternatively a Lineweaver–Burk plot which is concave downwards.[35] Therefore, another possible explanation for the curvature of the ΔA vs. ΔA/S plots is the presence (in freshly prepared microsomes) of a tightly bound substance(s) capable of causing a spectral change, with this substance acting as an alternative "substrate". It is unlikely that this alternative "substrate" could be phenobarbital and/or its metabolites as (1) preliminary experiments have shown that ΔA vs. ΔA/S plots still exhibit curvature when hepatic microsomes from control rats are used, and (2) Hart *et al.*[36] have indicated that phenobarbital is not detectable (less than 2 μg/g of tissue) in the liver of rats 12 hr after an intraperitoneal injection of phenobarbital (80 mg/kg). However, the presence of naturally occurring, tightly bound endogenous substrates (steroids?) cannot be ruled out at present.

A third possible reason for the observed curvature of the ΔA vs. ΔA/S plots is an allosteric effect; a relative decrease in the affinity of the binding site occurring with increasing amounts of added substrates (benzphetamine or aniline).

On microsomal storage the curvature of the ΔA vs. ΔA/S plots increased using type I substrates and decreased using type II substrates. This indicates that if (as postulated) different binding sites are associated with type I vs. type II substrate-induced spectral changes, aging affects these sites to different extents and probably in different ways.

Acknowledgements—We would like to thank Dr. P. W. O'Connell, Head, Biological Screening Office, The Upjohn Company, Kalamazoo, Michigan, for the gift of (+) *N*-benzyl-*N*, α-dimethylphenethylamine hydrochloride, and Don Shoeman, Department of Pharmacology, School of Medicine, University of Minnesota for the gift of ethyl isocyanide.

We also acknowledge the capable technical assistance of Mrs. Roberta Pohl, Mr. Douglas Rickert, Mr. Clarence Van Zee and Mrs. John Rios in various aspects of this work.

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